killed at 24 hours had positive lung homogenate cultures containing between 0.2 to 4.2 × 10^5 colony-forming units (cfu) per lung (mean, 5.1 × 10^4).

13. Animals were killed by an overdose of carbon dioxide, and the lungs were removed aseptically. Both lobes were placed in 5 ml of TSB in sterile glass homogenizers and mechanically homogenized for 30 s. To quantitate the degree of infection, serial dilutions in sterile saline were made and 0.1-ml amounts were plated onto cetrimide agar or tryptic soy agar (TSA), or both. These plates were incubated overnight at 37°C and mucoid *P. aeruginosa* colonies were enumerated. The remainder of the homogenates in TSB were incubated at 37°C for 5 days, after which they were subcultured onto TSA and cetrimide agar plates. The presence or absence of lung infection was determined by subculture of the total homogenate after the 5 days of incubation and examining these plates for mucoid *P. aeruginosa* colonies.
14. Quantitative counts of the number of bacteria per lung for animals in Table 1 ranged from 0.3 to 25.7 × 10^3. A statistical analysis based on the quantitative bacterial yields was not performed, since most animals with MEP-specific opsonizing antibody had sterile lung cultures.
15. For mice (virus- and antibody-free, C3H/HeN strain), the challenge inoculum was administered in 50-μl volumes. In all mouse experiments, five animals killed 24 hours after infection resulted in 100% of animals with lung homogenates yielding positive cultures for mucoid *P. aeruginosa* (mean 2.1 × 10^4 cfu per lung). Mouse lungs were homogenized in 1 ml of TSB, samples were removed for dilutions and enumeration, and TSB was added to achieve a volume of 5 ml. This suspension was incubated for 5 days and subcultured to determine the presence of mucoid *P. aeruginosa*. Quantitative counts ranged from 1.2 to 7.6 × 10^3 cfu per lung among infected animals.
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Regulation of Activity of a Transcriptional Anti-Terminator in *E. coli* by Phosphorylation in Vivo

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Expression of the *bgl* operon of *Escherichia coli* is regulated in vitro by phosphorylation and dephosphorylation of a positive regulatory protein, BglG, which functions in its nonphosphorylated state as a transcriptional antiterminator. The degree of phosphorylation of BglG in vivo was shown to be dependent on the cellular levels of BglF protein, which is both the BglG kinase and phosphatase. The degree of phosphorylation of BglG also depended on the presence or absence of a β-glucoside, the inducer of operon expression. Addition of inducer to cells in growth medium resulted in rapid dephosphorylation of phosphorylated BglG. The *bgl* operon is thus regulated by a sensory system that modulates gene expression by protein phosphorylation and dephosphorylation in response to the external levels of inducer.

IN THE LAST SEVERAL YEARS IT HAS become clear that protein phosphorylation is as widespread in bacteria as it is in eukaryotes (*1*). However, very few of the phosphorylated bacterial proteins have been characterized. Those that have been characterized include proteins involved in metabolic control, transport processes, bacteriophage infection, and, possibly, DNA replication (*2, 3*). Recent evidence from in vitro studies indicates that transient protein phosphorylation is involved in signal transduction systems that govern chemotaxis, nitrogen assimilation, and osmoregulation. These systems are regulated by pairs of proteins, one acting as sensor, the other as regulator (*4*). Only in the case of chemotaxis and nitrogen assimilation has it been possible to correlate in vitro phosphorylation of the regulatory protein by the sensor with in vivo function. In no case has protein phosphorylation been shown to be involved in regulation of gene expression in vivo.

Recently we reported that reversible protein phosphorylation regulates transcription, in vitro, of the β-glucoside utilization operon in *Escherichia coli* (*5*). This system also contains two regulatory proteins, though these are not members of the groups described above. BglF, a negative regulator of operon expression that also functions as a β-glucoside transporter, is a protein kinase, which, in the absence of β-glucoside inducer, phosphorylates the positive regulatory protein BglG and blocks its function as a transcriptional antiterminator. Upon addition of inducer, BglF dephosphorylates BglG, allowing it to function as a positive regulator of gene expression. On the basis of these studies, we proposed that the BglG protein, which acts as a transcriptional antiterminator (*6, 7*), exists in cells in a phosphorylated, inactive form in the absence of inducer and in a nonphosphorylated, active form in the presence of inducer (*5*).

To confirm that this is true in vivo and thus relevant to *bgl* operon induction, we attempted to show that the two forms of BglG are present in extracts prepared from growing cells. We used two-dimensional (2-D) gel electrophoresis to separate the two protein species from one another and from other proteins in the cell. This procedure involves isoelectric focusing in the first dimension and SDS–polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension. The products of an in vitro phosphorylation reaction (*5*) were used to determine the exact location of the two forms of BglG on 2-D gels (*8*). The phosphorylated and nonphosphorylated forms of BglG were separated from one another by this procedure (Fig. 1), the phosphorylated form being more acidic than the nonphosphorylated form. The phosphorylated form of BglG, labeled with [^{32}P]phosphate, was detected by autoradiography (Fig. 1A). Protein immunoblot analysis with antiserum to BglG identified both forms of BglG (Fig. 1B). Treatment of the proteins with alkaline phosphatase before gel analysis led to loss of the component identified as phosphorylated BglG, but had no effect on nonphosphorylated BglG (Fig. 1C).

To facilitate in vivo detection of BglG protein, we placed the *bglG* gene under control of the phage T7 promoter, making its expression independent of normal *Bgl* operon expression. Expression of the *bglG* gene was induced from the T7 promoter, in *bgl*$^+$ cells grown in the absence or presence of β-glucoside inducer (salicin). Cells were labeled with [^{35}S]methionine, in the presence or absence of rifampicin. Rifampicin prevents labeling of proteins encoded by genes transcribed by *E. coli* RNA polymer-

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ase, thus enabling almost exclusive labeling of BglG protein, a product of a gene transcribed by T7 RNA polymerase. The labeled proteins were analyzed on 2-D gels. Two forms of BglG were observed in vivo (Fig. 2); they had electrophoretic properties expected for the phosphorylated and nonphosphorylated forms. Approximately equal amounts of the two forms were present in the absence of inducer (Fig. 2, A and B), whereas in the presence of inducer the nonphosphorylated form predominated (Fig. 2, C and D). The use of rifampicin during labeling had no effect on the degree of phosphorylation of BglG and greatly simplified the analysis, BglG being the only labeled protein present in the gels. These results show that BglG is phosphorylated in vivo and that the extent of its phosphorylation was influenced by the presence of inducer in the growth medium.

To test whether BglG phosphorylation correlated with levels of BglF, the BglG kinase, in the cell, we analyzed the extent of BglG phosphorylation in cells producing different levels of BglF. We used wild-type *E. coli*, which is cryptic and noninducible for *bgl* operon expression (*bgl*^{o}) and thus produces very low levels of BglF (*9*); *E. coli* *bgl*$^{+}$, which is inducible for operon expression and has a higher basal expression of BglF than does the wild type (*9*); and *E. coli* *bgl*$^{+}$ that contained a plasmid carrying the *bglG* and *bglF* genes under control of the T7 promoter, which overproduces BglG and BglF. Analysis, by 2-D gel electrophoresis, of BglG produced in the three cell backgrounds in the absence of inducer indicated that the fraction of phosphorylated BglG increased with increased BglF production (Fig. 3). In the *bgl*^{o} background, the extent of BglG phosphorylation was low, nonphosphorylated BglG being the predominant species (Fig. 3A); in this background, in the presence of inducer, no phosphorylated BglG could be detected (*10*). In the *bgl*$^{+}$ background, about 50 to 60% of the protein was present in the phosphorylated form (Fig. 3B). Overproduction of BglF from a plasmid led to phosphorylation of approximately 90% of BglG in the cell (Fig. 3C). In this case, even though both genes were expressed from the T7 promoter, BglF was produced at considerably lower levels than BglG, as determined by SDS-PAGE analysis of the [^{35}S]methionine-labeled proteins (*11*). We conclude, from these experiments, that BglF acts in a catalytic rather than a stoichiometric way, and that the overall level of BglG phosphorylation is dependent on the BglF/BglG ratio.

The results presented in Fig. 2 demonstrate that when cells are grown in the presence of β-glucoside inducer, the amount of phosphorylated BglG that could be detected was negligible. From this type of experiment it is difficult to determine whether inducer prevents BglG phosphoryl-

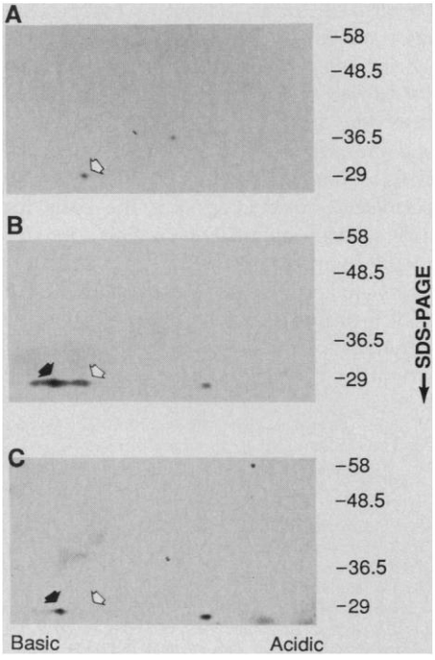

Fig. 1. Separation of phosphorylated and nonphosphorylated BglG on a 2-D gel. The BglG protein was labeled with ^{32}P in an in vitro reaction mixture containing BglF, enzyme I, HPr, and [^{32}P]phosphoenolpyruvate as previously described (*5*). The products of the reaction were fractionated by 2-D gel electrophoresis, involving isoelectric focusing in the first dimension and SDS-PAGE in the second dimension. (**A**) An autoradiogram of the gel. (**B**) Protein immunoblot analysis with antiserum to BglG. (**C**) The same as (B) except that the protein sample was treated with alkaline phosphatase before electrophoresis. The closed arrows indicate the position of nonphosphorylated BglG; the open arrows indicate the position of phosphorylated BglG. Molecular masses of protein standards are given in kilodaltons. Proteins were solubilized in Garrel's sample buffer (*12*) and analyzed by 2-D gel electrophoresis (*13, 14*). Protein extracts were treated in sample buffer with *E. coli* alkaline phosphatase (Sigma) (0.5 μg/ml) for 5 min before electrophoresis. For protein immunoblot analysis, proteins were electrophoretically transferred (*15*) to Immobilon-P membranes (Millipore). The preparation of antiserum to BglG was described previously (*5*).

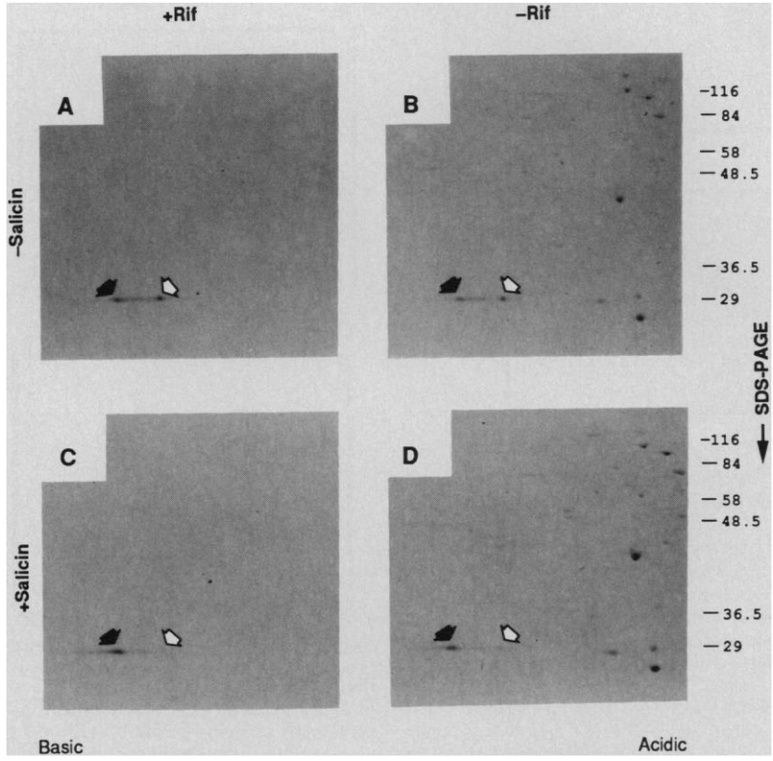

Fig. 2. BglG protein is phosphorylated in vivo; influence of β-glucosides on the state of phosphorylation. *Escherichia coli* K38 *bgl*$^{+}$ cells containing plasmids pT7FH-G (with the *bglG* gene under T7 promoter control) and pGP1-2 (with a heat-inducible T7 RNA polymerase gene) were grown in the absence (**A** and **B**) and presence (**C** and **D**) of 0.4% salicin. BglG expression was induced by shifting the culture to 42°, and the cells were labeled with [^{35}S]methionine in the presence (A and C) or absence (B and D) of rifampicin. Labeled proteins were fractionated on 2-D gels, and the gels were analyzed by autoradiography. The closed arrows indicate the position of nonphosphorylated BglG; the open arrows indicate the position of phosphorylated BglG. Molecular masses of protein standards are given in kilodaltons. The bacterial strain and plasmids pT7FH-G and pGP1-2 and the methods used for growth, induction and [^{35}S]methionine labeling have been described previously (*5, 16*). Cells were harvested after labeling and washed with saline; the proteins were solubilized and analyzed as described in Fig. 1.

ation or can actually lead to dephosphorylation of this protein, as suggested by in vitro studies (*5*). To answer this question, we carried out pulse-chase experiments in which inducer was added only after BglG protein was phosphorylated in the cell (Fig. 4). When cells overproducing BglF and BglG were pulse-labeled with [^{35}S]methionine in the absence of inducer, BglG was present almost solely in the phosphorylated form (Fig. 4A). Addition of unlabeled methionine to the pulse-labeled cells had no effect on the level of phosphorylated BglG (Fig. 4B). However, when β-glucoside inducer was added to the cells together with unlabeled methionine, all of the phosphorylated BglG was converted to the nonphosphorylated form of this protein (Fig. 4C). Thus dephosphorylation of BglG occurs in vivo upon addition of β-glucoside to the growth medium.

The results presented in this paper support the mechanism we proposed for the regulation of *bgl* operon expression, namely, that BglG antitermination activity is regulated by phosphorylation (*5*). We show here that BglG is phosphorylated in vivo in the absence of inducer and is dephosphorylated upon addition of inducer to the growth medium. In all of the above experiments, BglG protein was produced at higher than normal levels to facilitate its detection. Nevertheless, it was efficiently phosphorylated by BglF and also rapidly dephosphorylated upon addition of inducer.

The main reasons for the lack of direct proof, so far, for the involvement of transient phosphorylation events in processes like chemotaxis, nitrogen assimilation, and osmoregulation are the low abundance and lability of the protein intermediates containing unstable phospho–amino acids, making in vivo detection difficult. We solved the problem of low abundance by overproducing the regulatory protein. The protein was labeled with [^{35}S]methionine, rather than ^{32}P-labeled inorganic phosphate, to allow detection of both the nonphosphorylated and phosphorylated forms. The difference in charge between the protein and its phosphorylated counterpart was the basis for their separation on 2-D gels. This approach should be applicable to the analysis of phosphorylated protein intermediates involved in both prokaryotic and eukaryotic regulatory systems.

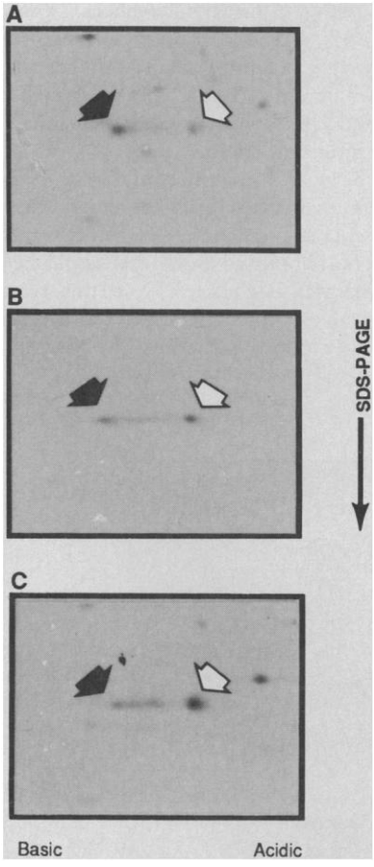

Fig. 3. The effect of BglF concentration on BglG phosphorylation in vivo. Cells, grown in all cases at 30°C in the absence of inducer, were induced for BglG synthesis by shifting cultures to 42°C and labeled with [^{35}S]methionine. The bacterial strains used were (**A**) *E. coli* K38 *bgl*^{o} (uninducible for *bgl* operon expression) containing plasmids pT7FH-G and pGP1-2; (**B**) *E. coli* K38 *bgl*$^{+}$ containing plasmids pT7FH-G and pGP1-2; and (**C**) *E. coli* K38 *bgl*$^{+}$ containing plasmids pT7OAC-GF (with *bglG* and *bglF* under T7 promoter control) and pGP1-2. ^{35}S-labeled proteins were fractionated by 2-D gel electrophoresis. The closed arrows indicate the position of nonphosphorylated BglG; the open arrows show the position of phosphorylated BglG. Plasmid pT7OAC-GF was constructed by cloning a 3453-bp Sna BI–Pvu II fragment, carrying the *bglF* and *bglG* genes from pOAC1 into the Hinc II site on plasmid pT712 (Bethesda Research Laboratories). The cloned fragment was downstream of the T7 promoter, with the *bglG* gene proximal to the promoter. pOAC1 is a pBR322 derivative carrying the entire *bgl* operon.

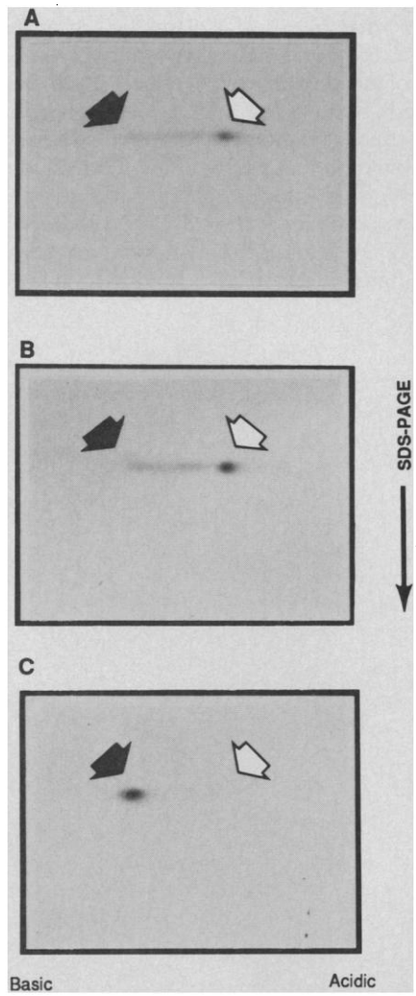

Fig. 4. β-Glucosides stimulate dephosphorylation of BglG in vivo. *Escherichia coli* K38 *bgl*$^{+}$ cells containing plasmids pT7OAC-GF and pGP1-2 were grown in the absence of inducer, shifted to 42°C to induce BglG and BglF synthesis, and then labeled by incubation with [^{35}S]methionine for 10 min. (**A**); labeled by incubation with [^{35}S]methionine for 10 min and then further incubated for 20 min with excess unlabeled methionine (**B**); or labeled as in (B) except that 0.4% salicin was added at the same time as the unlabeled methionine (**C**). The closed arrows indicate the position of nonphosphorylated BglG; the open arrows indicate the position of phosphorylated BglG.

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